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(71) Applicant (for all designated States except US): GLAXO GROUP LIMITED [GB/GB]; Glaxo Wellcome plc, Glaxo Wellcome House, Berkeley Avenue, Greenford, Middlesex UB6 0NN (GB).

(72) Inventor; and
(75) Inventor/Applicant (for US only): BALU, Palani

(74) Agent: STRACKER, Elaine, C.; Affymax Research Institute, 4001 Miranda Avenue, Palo Alto, CA 94304 (US).

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(54) Title: NOVEL PEPTIDE DIMERS AS AGONISTS OF THE ERYTHROPOIETIN (EPO) RECEPTOR, AND ASSOCIATED METHODS OF SYNTHESIS AND USE

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(57) Abstract: Novel peptide dimers are provided that bind and activate the erythropoietin receptor (EPO-R) or otherwise act as an EPO agonist. The novel compounds have a first peptide chain R¹ and a second peptide chain R², wherein R¹ and R² may be the same or different, and are linked through a linking moiety. R¹ is approximately 10 to 40 amino acid residues in length and comprises the sequence X₃X₄X₅GPX₆TX₇X₈X₉ (SEQ ID NO: 1) wherein X₃ is C or Hoc, X₄ is R, H, L or W, X₅ is M, F, I or nor-leucine (J), X₆ is any one of the 20 genetically coded L-amino acids or J, X₇ is W, 1-naphthylalanine (B) or 2-naphthylalanine (U), X₈ is D, E, I, L or V, and X₉ is C or Hoc. Similarly, R² comprises the sequence X'₃X'₄X'₅GPX'₆TX'₇X'₈X'₉ (SEQ ID NO: 2) wherein X'₃ is C or Hoc, X'₄ is R, H, L or W, X'₅ is M, F, I or J, X'₆ is any one of the 20 genetically coded L-amino acids or J, X'₇ is W, B or U, X'₈ is D, E, I, L or V and X'₉ is C or Hoc. Methods for synthesizing the compounds are provided as well, as are pharmaceutical compositions and methods of use.

NOVEL PEPTIDE DIMERS AS AGONISTS OF THE ERYTHROPOIETIN (EPO)
RECEPTOR, AND ASSOCIATED METHODS OF SYNTHESIS AND USE

TECHNICAL FIELD

5 The present invention provides novel compounds that bind to and activate the erythropoietin (EPO) receptor or otherwise act as EPO agonists. The invention additionally relates to methods for synthesizing the novel compounds, methods of using the novel compounds, and pharmaceutical compositions containing a compound of the invention as the active agent. The invention has application in the fields of biochemistry and medicinal
10 chemistry and particularly provides EPO agonists for use in the treatment of human disease.

BACKGROUND

Erythropoietin (EPO) is a glycoprotein hormone with 165 amino acids, 4 glycosylation sites on amino-acid positions 24, 38, 83, and 126, and a molecular weight of
15 about 34,000. It is initially produced as a precursor protein with a signal peptide of 23 amino acids. EPO can occur in three forms: α , β , and asialo. The α and β forms differ slightly in the carbohydrate components, but have the same potency, biological activity, and molecular weight. The asialo form is an α or β form with the terminal carbohydrate (sialic acid) removed. The DNA sequences encoding EPO have been reported. See, Lin (1987) U.S. Pat. No. 4,703,008.

20 EPO stimulates mitotic division and the differentiation of erythrocyte precursor cells and thus ensures the production of erythrocytes. It is produced in the kidney when hypoxic conditions prevail. During EPO-induced differentiation of erythrocyte precursor cells, there is induction of globin synthesis and increases in the synthesis of the heme complex and in the number of ferritin receptors. This makes it possible for the cell to take on more iron and
25 synthesize functional hemoglobin. Hemoglobin in mature erythrocytes binds oxygen. Thus, the erythrocytes and the hemoglobin contained in them play a key part in supplying the body with oxygen. The complex processes which have been described are initiated by the interaction of EPO with an appropriate receptor on the cell surface of the erythrocyte precursor cells. See, e.g., Gruber and Krantz (1978) *Ann. Rev. Med.* 29:51-66.

30 EPO is present in very low concentrations in plasma when the body is in a healthy state wherein tissues receive sufficient oxygenation from the existing number of erythrocytes.

This normal low concentration is enough to stimulate replacement of red blood cells which are lost normally through aging.

The amount of EPO in the circulation is increased under conditions of hypoxia when oxygen transport by blood cells in the circulation is reduced. Hypoxia may be caused by loss of 5 large amounts of blood through hemorrhage, destruction of red blood cells by over-exposure to radiation, reduction in oxygen intake due to high altitudes or prolonged unconsciousness, or various forms of anemia. In response to tissues undergoing hypoxic stress, EPO will increase red blood cell production by stimulation of proliferation of erythroid progenitor cells. When the 10 number of red blood cells in circulation is greater than needed for normal tissue oxygen requirements, EPO in circulation is decreased.

Because EPO is essential in the process of red blood cell formation, the hormone has potentially useful applications in both the diagnosis and the treatment of blood disorders characterized by low or defective red blood cell production. Recent studies have provided a 15 basis for the projection of efficacy of EPO therapy in a variety of disease states, disorders, and states of hematologic irregularity, including: beta-thalassemia (see, Vedovato et al. (1984) *Acta Haematol.* 71:211-213); cystic fibrosis (see Vichinsky et al. (1984) *J. Pediatric.* 105:15-21; pregnancy and menstrual disorders (see Cotes et al. (1993) *Brit. J. Obstet. Gynecol.* 90:304-311; early anemia of prematurity (see Haga et al. (1983) *Acta Pediatr. Scand.* 72:827-831); spinal cord injury (see Claus-Walker et al. (1984) *Arch. Phys. Med. Rehabil.* 65:370-374); space flight (see Dunn et al. (1984) *Eur. J. Appl. Physiol.* 52:178-182); acute 20 blood loss (see Miller et al. (1982) *Brit. J. Haematol.* 52:545-590); aging (see Udupa et al. (1984) *J. Lab. Clin. Med.* 103:574-580 and 581-588 and Lipschitz et al. (1983) *Blood* 63:502-509; various neoplastic disease states accompanied by abnormal erythropoiesis (see Dainiak et al. (1983) *Cancer* 5:1101-1106 and Schwartz et al. (1983) *Otolaryngol.* 109:269-272); and renal insufficiency (see Eschbach et al. (1987) *N. Eng. J. Med.* 316:73-78).

Purified, homogeneous EPO has been characterized. See Hewick, U.S. Pat. No. 4,677,195. A DNA sequence encoding EPO was purified, cloned and expressed to produce synthetic polypeptides with the same biochemical and immunological properties. A recombinant EPO molecule with oligosaccharides identical to those on the natural material has 30 also been produced. See, Sasaki et al. (1987) *J. Biol. Chem.* 262:12059-12076.

Despite the availability of purified recombinant EPO, little is known concerning the mechanism of EPO-induced erythroblast proliferation and differentiation. The specific

interaction of EPO with progenitors of immature red blood cells, platelets, and megakaryocytes remains to be characterized. This is due, at least in part, to the small number of surface EPO receptor molecules on normal erythroblasts and on the erythroleukemia cell line. See, Krantz and Goldwasser (1984) *Proc. Natl. Acad. Sci. USA* 81:7574-7578; Branch et al. (1987) *Blood* 69:1782-1785; Mayeux et al. (1987) *FEBS Letters* 211:229-233; Mufson and Gesner (1987) *Blood* 69:1485-1490; Sakaguchi et al. (1987) *Biochem. Biophys. Res. Commun.* 146:7-12; Sawyer et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:3690-3694; Sawyer et al. (1987) *J. Biol. Chem.* 262:5554-5562; and Todokoro et al. (1988) *Proc. Natl. Acad. Sci. USA* 84:4126-4130.

Cross-linked complexes between radioiodinated EPO and cell surface proteins suggest that the cell surface proteins comprise two polypeptides having approximate molecular weights of 85,000 daltons and 100,000 daltons, respectively. More recently, the two cross-linked complexes have been subjected to V8 protease digestion and have been found to have identical peptide fragments, suggesting that the two EPO-binding polypeptides may be products of the same or very similar genes. See, Sawyer et al. (1988) *supra*. Most cell surface binding studies, however, have revealed a single class of binding sites, averaging 300 to 600 per cell surface, with a K_d of approximately 800 pM (picomolar). See, Sawyer et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:3690-3694. However, EPO-responsive splenic erythroblasts, prepared from mice injected with the anemic strain (FVA) of the Friend leukemia virus, demonstrate a high and a low affinity binding site with dissociation constants of 100 pM and 800 pM, respectively. See, Sawyer et al. (1987) *J. Biol. Chem.* 262:5554-5562 and Landschulz (1989) *Blood* 73:1476-1478. The DNA sequences and encoded peptide sequences for murine and human EPO receptor proteins have been described. See, D'Andrea et al., PCT Patent Publication No. WO 90/08822 (published 1990).

The availability of cloned genes for the EPO receptor (EPO-R) facilitates the search for agonists and antagonists of this important receptor. The availability of the recombinant receptor protein allows the study of receptor-ligand interaction in a variety of random and semi-random peptide diversity generation systems. These systems include the "peptides on plasmids" system described in U.S. patent application Ser. No. 778,233, filed Oct. 16, 1991, issued as U.S. Pat. No. 5,270,170, the "peptides on phage" system described in U.S. patent application Ser. No. 718,577, issued as U.S. Pat. No. 5,432,018 and in Cwirla et al., Aug. 1990, *Proc. Natl. Acad. Sci. USA* 87:6378-6382, the "encoded synthetic library" (ESL) system described in U.S. patent application Ser. No. 946,239, filed Sep. 16, 1992, which is a

continuation-in-part application of Ser. No. 762,522, filed Sep. 18, 1991, now abandoned, the "very large scale immobilized polymer synthesis" system described in U.S. patent application Ser. No.492,462, filed Mar. 7, 1990, now U.S. Pat. No. 5,143,854, and those systems described in PCT patent publication No. 90/15070, published Dec. 13, 1990, U.S. patent application Ser.

5 No. 624,120, filed Dec. 6, 1990, now abandoned, Fodor et al., 15 Feb. 1991, *Science* 251:767-773, Dower and Fodor, 1991, *Ann. Rep. Med. Chem.* 26:271-180, and U.S. patent application Ser. No. 805,727, filed Dec. 6, 1991, now U.S. Pat. No. 5,424,186.

Novel peptides that interact with the EPO-R have been discovered, and have been disclosed in U.S. Patent No. 5,773,569 to Wrighton et al. The Wrighton et al. peptides are 10 generally single polypeptide chains about 10 to 40 amino acids in length, and contain a pair of cysteine residues separated by eight amino acids. These EPO-R-binding peptides were found to bind to or otherwise interact with the EPO-R, and were thus presumed to be useful for studying the biological activities of the EPO-R and for treatment of disease involving a deficiency of EPO. A related patent, U.S. Patent No. 5,830,851 to Wrighton et al., pertains more specifically 15 to therapeutic applications of the aforementioned peptides, and focuses on the use of the compounds to treat disorders such as end-stage renal failure or dialysis, anemia associated with AIDS, autoimmune diseases, malignancies, and chronic inflammatory disease, beta-thalassemia, cystic fibrosis, spinal cord injury, acute blood loss, aging, and neoplastic disease states accompanied by abnormal erythropoiesis.

20 There remains a need, however, for highly active compounds that bind very specifically to the EPO-R, both for studies of the important biological activities mediated by the receptor and for treatment of diseases, disorders and conditions associated with an EPO deficiency. There remains a further need for improved synthetic methods for the production of such compounds. The present invention provides such compounds and methods, and also 25 provides pharmaceutical compositions and methods for using the compounds as therapeutic agents.

SUMMARY OF THE INVENTION

In one embodiment, the invention provides compounds in the form of peptide dimers 30 that bind to and activate the EPO-R or otherwise behave as EPO agonists. The compounds have a first peptide chain R¹ and a second peptide chain R², wherein R¹ and R² may be the same or different, and are linked through a linking moiety. R¹ is approximately 10 to 40 amino acid

residues in length and comprises a core sequence of amino acids $X_3X_4X_5GPX_6TX_7X_8X_9$ (SEQ ID NO: 1) wherein each amino acid is indicated by standard one-letter abbreviation, X_3 is C or homocysteine (Hoc), X_4 is R, H, L or W, X_5 is M, F, I or nor-leucine (J), X_6 is selected from any one of the 20 genetically coded L-amino acids and J, X_7 is W, 1-naphthylalanine (also referred to herein as "1-Nal" or B) or 2-naphthylalanine (also referred to herein as "2-Nal" or U), X_8 is D, E, I, L or V, and X_9 is C or Hoc. R^2 is also approximately 10 to 40 amino acid residues in length and, similarly, comprises a core sequence of amino acids $X'_3X'_4X'_5GPX'_6TX'_7X'_8X'_9$ (SEQ ID NO: 2) wherein each amino acid is indicated by standard one-letter abbreviation, X'_3 is C or Hoc, X'_4 is R, H, L or W, X'_5 is M, F, I or J, X'_6 is selected from any one of the 20 genetically coded L-amino acids and J, X'_7 is W, B or U, X'_8 is D, E, I, L or V, and X'_9 is C or Hoc. The linker moiety between R^1 and R^2 is preferably although not necessarily a C_{1-12} linking moiety optionally terminated with one or two -NH- linkages and optionally substituted at one or more available carbon atoms with a lower alkyl substituent. In addition, a β -alanine residue may be present between R^1 and the linking moiety, or between R^2 and the linking moiety, or both. Generally, although not necessarily, either (a) X_3 and X_9 are linked by a disulfide bond and X'_3 and X'_9 are linked by a disulfide bond, (b) X_3 and X'_9 are linked by a disulfide bond and X'_3 and X_9 are linked by a disulfide bond, or (c) X_3 and X'_3 are linked by a disulfide bond and X'_9 and X_9 are linked by a disulfide bond.

More preferably, R^1 comprises a core sequence of amino acids $YX_2X_3X_4X_5GPX_6TBX_8X_9X_{10}$ (SEQ ID NO: 3) wherein X_2 , X_6 and X_{10} are independently selected from any one of the 20 genetically coded L-amino acids, X_3 is C or Hoc, X_4 is R, H or L, X_5 is M, F or I, X_8 is D, E, I, L or V, and X_9 is C or Hoc, R^2 comprises a core sequence of amino acids $YX'_2X'_3X'_4X'_5GPX'_6TBX'_8X'_9X'_{10}$ (SEQ ID NO: 4) wherein X'_2 , X'_6 and X'_{10} are independently selected from any one of the 20 genetically coded L-amino acids, X'_3 is C or Hoc, X'_4 is R, H or L, X'_5 is M, F or I, X'_8 is D, E, I, L or V, and X'_9 is C or Hoc, and the dimer contains two disulfide bonds.

In a most preferred embodiment, R^1 comprises a core sequence of amino acids $X_1YX_2CX_4X_5GPX_6TBX_8CX_{10}X_{11}X_{12}$ (SEQ ID NO: 5) wherein X_1 , X_2 , X_6 , X_{11} and X_{12} are independently selected from any one of the 20 genetically coded L-amino acids, X_4 is R or H, X_5 is M or F, X_8 is D, E, I, L or V, and X_{10} is R; R^2 comprises a core sequence of amino acids $X'_1YX'_2CX'_4X'_5GPX'_6TBX'_8CX'_{10}X'_{11}X'_{12}$ (SEQ ID NO: 6) wherein X'_1 , X'_2 , X'_6 , X'_{11} and X'_{12} are independently selected from any one of the 20 genetically coded L-amino acids, X'_4 is R or

H, X₅' is M or F, X₈' is D, E, I, L or V, and X₁₀' is R, wherein R¹ and R² are identical; the thiol functionalities of X₃ and X₉ are linked to form an intramolecular disulfide bond within the R¹ and, similarly, the thiol functionalities of X₃' and X₉' are linked to form an intramolecular disulfide bond within R²; the linker comprises -NH-R³-NH- wherein R³ is lower (C₁₋₆) alkylene

5 substituted with a functional group such as a carboxyl group that enables binding to another molecular moiety (e.g., as may be present on the surface of a solid support), and is optionally substituted with a lower alkyl group; and a β -alanine residue is present between R¹ and the linker and/or between R² and the linker. Optimally, the linker is a lysine residue.

According to some embodiments of this invention, two or more, and preferably

10 between two and six amino acid residues, independently selected from any of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids, will be coupled to the free (amino) terminus of R¹ and/or R², i.e., the terminus of the peptide chain that is not bound to the linker. In addition, or in the alternative, such amino acid residues will be present at the opposing terminus of R¹ and/or R², i.e., between R¹ and/or R² and the β -alanine group, if one is present, 15 or between R¹ and/or R² and the linker moiety, if no β -alanine group is present. For example, the sequence GG will often be appended to termini of the core sequences for ease in synthesis of the peptides. Other modifications are also possible, including capping or otherwise modifying the free amino terminus of R¹ and/or R², replacement of one or more of the naturally occurring, genetically encoded amino acids with a synthetic amino acid, peptide phosphorylation, 20 modification of an amino acid side chain, and the like. Particularly preferred modification comprises covalent attachment of polyethylene glycol moieties at the N-terminus of both R¹ and R².

The invention also provides a method for synthesizing the aforementioned dimer compounds wherein each of R¹ and R² independently contains a cyclic moiety deriving from an 25 intramolecular disulfide bond formed between two cysteine or homocysteine residues. The method is preferably a one-step oxidation process which preferentially results in the desired compound, i.e., a dimer with each peptide chain containing an intramolecular cyclic structure, and minimizes the less desirable (i.e., less active) products in which disulfide bridges are present linking R¹ and R².

30 The present invention also provides methods for treating disease involving a deficiency of EPO utilizing the novel compounds of the invention, and further provides

pharmaceutical compositions comprising one or more compounds of the invention and a physiologically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figures 1-1, 1-2 and 1-3 provide the sequences of representative R¹ and R² peptide chains contained within the dimers of the invention.

Figures 2-1 and 2-2 illustrate the synthetic scheme for preparation of a dimeric peptide of the invention using sequential synthesis of the chains and regioselective oxidation.

10 Figure 3 illustrates a preferred synthetic scheme for preparation of a dimeric peptide of the invention, wherein the two peptide chains are synthesized simultaneously and a one-step "oxidative folding" technique is used to promote cyclization within each peptide chain.

Figure 4 illustrates the molecular structures of the three isomers that can result from dimer oxidation in the absence of a regioselective process.

15 Figure 5 illustrates the biological activity of various dimeric peptide analogs of the invention, as explained in Example 14.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS AND OVERVIEW:

It is to be understood that unless otherwise indicated, this invention is not limited to 20 specific peptide sequences, molecular structures, pharmaceutical compositions, methods of synthesis, or the like, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

It must be noted that, as used in the specification and the appended claims, the 25 singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a novel compound" in a pharmaceutical composition means that more than one of the novel compounds can be present in the composition, reference to "a pharmaceutically acceptable carrier" includes combinations of such carriers, and the like.

30 In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings:

Amino acid residues in peptides are abbreviated as follows: Phenylalanine

is Phe or F; Leucine is Leu or L; Isoleucine is Ile or I; Methionine is Met or M; Valine is Val or V; Serine is Ser or S; Proline is Pro or P; Threonine is Thr or T; Alanine is Ala or A; Tyrosine is Tyr or Y; Histidine is His or H; Glutamine is Gln or Q; Asparagine is Asn or N; Lysine is Lys or K; Aspartic Acid is Asp or D; Glutamic Acid is Glu or E; Cysteine is Cys or C; Tryptophan 5 is Trp or W; Arginine is Arg or R; and Glycine is Gly or G. In addition, the letter "B" is used to refer to 1-naphthylalanine (also sometimes referred to herein as "1-Nal"), the letter "U" is used to refer to 2-naphthylalanine (also sometimes referred to herein as "2-Nal"), and the letter "J" is used to refer to nor-leucine.

Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, 10 unnatural amino acids such as α,α -disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for compounds of the present invention. Examples of unconventional amino acids include: β -alanine, 1-naphthylalanine, 2-naphthylalanine, 3-pyridylalanine, 4-hydroxyproline, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylsine, nor-leucine, and other 15 similar amino acids and imino acids (e.g., 4-hydroxyproline).

"Peptide" or "polypeptide" refers to a polymer in which the monomers are alpha amino acids joined together through amide bonds. Peptides are two or often more amino acid monomers long.

The term "dimer" as in a peptide "dimer" refers to a compound in which two peptide 20 chains are linked; generally, although not necessarily, the two peptide chains will be identical and are linked through a linking moiety covalently bound to the carboxyl terminus of each chain.

"Agonist" refers to a biologically active ligand which binds to its complementary biologically active receptor and activates the latter either to cause a biological response in the 25 receptor, or to enhance preexisting biological activity of the receptor.

"Pharmaceutically acceptable salts" refer to the non-toxic alkali metal, alkaline earth metal, and ammonium salts commonly used in the pharmaceutical industry including the sodium, potassium, lithium, calcium, magnesium, barium, ammonium and protamine zinc salts, which are prepared by methods well known in the art. The term also includes non-toxic acid 30 addition salts, which are generally prepared by reacting the compounds of this invention with a suitable organic or inorganic acid. Representative salts include the hydrochloride,

hydrobromide, sulfate, bisulfate, acetate, oxalate, valerate, oleate, laurate, borate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, napsylate and the like.

"Pharmaceutically or therapeutically effective dose or amount" refers to a dosage level sufficient to induce a desired biological result. That result can be alleviation of the signs, 5 symptoms, or causes of a disease, or any other desired alteration of a biological system. Preferably, this dose or amount will be sufficient to stimulate the EPO-R and, thus, alleviate the symptoms associated with a deficiency of EPO, or a defective or low red blood population *in vivo*.

The term "treat" as in "to treat a disease" is intended to include any means of treating 10 a disease in a mammal, including (1) preventing the disease, i.e., avoiding any clinical symptoms of the disease, (2) inhibiting the disease, that is, arresting the development or progression of clinical symptoms, and/or (3) relieving the disease, i.e., causing regression of clinical symptoms.

The terms "disease," "disorder" and "condition" are used interchangeably herein to 15 refer to a physiological state associated with a deficiency of EPO.

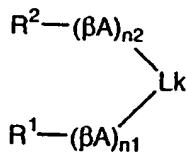
"Optional" or "optionally" means that the subsequently described circumstance may or may not occur, so that the description includes instances where the circumstance occurs and instances where it does not.

By "pharmaceutically acceptable carrier" is meant a material which is not biologically 20 or otherwise undesirable, i.e., the material may be administered to an individual along with the selected active agent without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. Similarly, a "pharmaceutically acceptable" salt of a novel compound as provided herein is a salt or ester which is not biologically or otherwise undesirable.

25

II. THE NOVEL COMPOUNDS:

In a first embodiment, the invention provides compounds that bind to and activate the EPO-R or otherwise behave as an EPO agonist. The compounds have the structure of formula (I)



(I)

wherein R^1 , R^2 , $n1$, $n2$ and Lk are defined as follows.

R^1 is a peptide approximately 10 to 40 amino acid residues in length that comprises a core sequence of amino acids $\text{X}_3\text{X}_4\text{X}_5\text{GPX}_6\text{TX}_7\text{X}_8\text{X}_9$ (SEQ ID NO: 1) wherein each amino acid is indicated by standard one-letter abbreviation; X_3 is C or Hoc; X_4 is R, H, L or W; X_5 is M, F, 5 I or J; X_6 is selected from any one of the 20 genetically coded L-amino acids and J; X_7 is W, B or U; X_8 is D, E, I, L or V; and X_9 is C or Hoc.

R^2 is also a peptide approximately 10 to 40 amino acid residues in length and, similarly, comprises a core sequence of amino acids $\text{X}'_3\text{X}'_4\text{X}'_5\text{GPX}'_6\text{TX}'_7\text{X}'_8\text{X}'_9$ (SEQ ID NO: 2) wherein each amino acid is indicated by standard one-letter abbreviation; X'_3 is C or Hoc; X'_4 10 is R, H, L or W; X'_5 is M, F, I or J; X'_6 is selected from any one of the 20 genetically coded L-amino acids and J; X'_7 is W, B or U; X'_8 is D, E, I, L or V; and X'_9 is C or Hoc.

The β -alanine (" βA ") residues may or may not be present, meaning that $n1$ and $n2$ are independently zero or 1.

Lk is a C_{1-12} linking moiety optionally terminated with one or two -NH- linkages and 15 optionally substituted at one or more available carbon atoms with a lower alkyl substituent. Preferably the linker Lk comprises -NH- R^3 -NH- wherein R^3 is lower (C_{1-6}) alkylene substituted with a functional group such as a carboxyl group or an amino group that enables binding to another molecular moiety (e.g., as may be present on the surface of a solid support), and is 20 optionally substituted with a lower alkyl group. Optimally, the linker Lk is a lysine residue or lysine amide, i.e., a lysine residue wherein the carboxyl group has been converted to an amide moiety -CONH₂.

The cysteine or homocysteine residues at X_3 , X_9 , X'_3 , and X'_9 may contain free thiol groups, or disulfide bonds may be present linking these residues. Typically, the dimer contains two disulfide bonds, wherein either (a) X_3 and X_9 are linked by a disulfide bond and X'_3 and X'_9 25 are linked by a disulfide bond, (b) X_3 and X'_9 are linked by a disulfide bond and X'_3 and X_9 are linked by a disulfide bond, or (c) X_3 and X'_3 are linked by a disulfide bond and X'_9 and X_9 are linked by a disulfide bond. Option (a) is preferred, such that each peptide chain contains a cyclic group.

In addition, the amino terminus of R^1 (i.e., the terminus opposite the carboxyl terminus, which is bound directly or indirectly to the linker), the amino terminus of R^2 , or both, may be modified by acetylation (with, for example, acetic acid, or a halogen-substituted acetic acid), or by coupling to a polyethylene glycol moiety.

5 In a more preferred embodiment, with respect to the peptide chains, R^1 comprise a core sequence of amino acids $YX_2X_3X_4X_5GPX_6TBX_8X_9X_{10}$ (SEQ ID NO: 3) wherein X_2 , X_6 and X_{10} are independently selected from any one of the 20 genetically coded L-amino acids, X_3 is C or Hoc, X_4 is R, H or L, X_5 is M, F or I, X_8 is D, E, I, L or V, and X_9 is C or Hoc, and R^2 comprise a core sequence of amino acids $YX'_2X'_3X'_4X'_5GPX'_6TBX'_8X'_9X'_{10}$ (SEQ ID NO: 4) 10 wherein X'_2 , X'_6 and X'_{10} are independently selected from any one of the 20 genetically coded L-amino acids, X'_3 is C or Hoc, X'_4 is R, H or L, X'_5 is M, F or I, X'_8 is D, E, I, L or V, and X'_9 is C or Hoc. It is also preferred, although not essential, that R^1 and R^2 be identical.

15 In a most preferred embodiment: R^1 comprises a core sequence of amino acids $X_1YX_2CX_4X_5GPX_6TBX_8CX_{10}X_{11}X_{12}$ (SEQ ID NO: 5) wherein X_1 , X_2 , X_6 , X_{11} and X_{12} are independently selected from any one of the 20 genetically coded L-amino acids, X_4 is R or H, X_5 is M or F, X_8 is D, E, I, L or V, and X_{10} is R; R^2 comprises a core sequence of amino acids $X'_1YX'_2CX'_4X'_5GPX'_6TBX'_8CX'_{10}X'_{11}X'_{12}$ (SEQ ID NO: 6) wherein X'_1 , X'_2 , X'_6 , X'_{11} and X'_{12} are independently selected from any one of the 20 genetically coded L-amino acids, X'_4 is R or H, X'_5 is M or F, X'_8 is D, E, I, L or V, and X'_{10} is R; R^1 and R^2 are identical; the two cysteine 20 residues in each of R^1 and R^2 are linked to form a disulfide bond (so that each peptide chain contains a cyclic group); no lysine residues are present; and polyethylene glycol molecules are coupled to both R^1 and R^2 at the amino terminus of each chain.

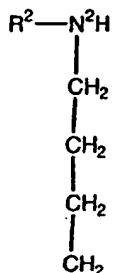
25 Relative to the EPO-R agonists that have been previously disclosed, for example in U.S. Patent No. 5,773,659 to Wrighton et al., the dimers of the invention are surprisingly more active. This appears to be due in part to the advantageous substitution of a 1-naphthylalanine residue (B) for tryptophan (W) (as disclosed in the aforementioned patent) at X_7 and X'_7 , which provides for a six-fold more potent EPO agonist, in part to the two amino-terminal polyethylene glycol molecules in preferred compounds, which have been found to dramatically increases serum half-life and biological activity, and in part to the dimeric structure itself. The omission 30 of lysine residues, such as at X_{10} and X'_{10} , is also advantageous in that N-terminal specific modification is facilitated, i.e., no other amino groups are present in the peptide chains.

-12-

When the linker L_K is lysine amide and $n1$ and $n2$ are zero, the dimer may be illustrated structurally as follows:

5

(II)

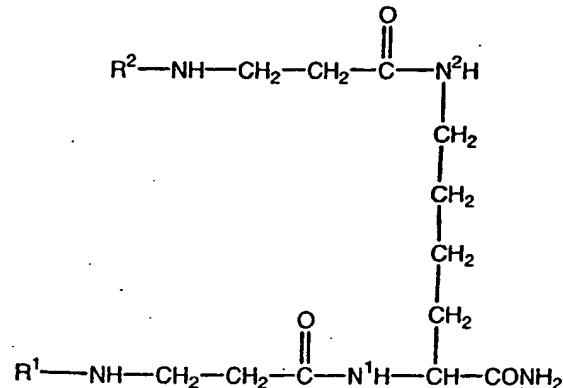


In formula (II), N^2 represents the nitrogen atom of lysine's ϵ -amino group and N^1 represents the nitrogen atom of lysine's α -amino group.

10 Similarly, when L_K is lysine amide and $n1$ and $n2$ are both 1, the dimer has the structure of formula (III)

15

(III)

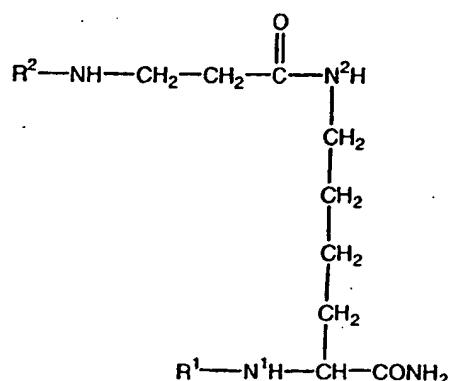


20

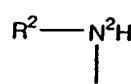
Analogously, structure (IV) represents a dimer herein wherein L_K is lysine amide, $n1$ is zero and $n2$ is 1, while structure (V) represents a dimer wherein L_K is lysine amide, $n1$ is 1 and $n2$ is zero:

25

(IV)



30



(V)

5

Examples of particularly preferred R¹ and R² sequences include, but are not limited to, the following:

10 GGTYSCHFGPLTBVCRPQGG (SEQ ID NO: 7);
GGTYSCHFGPLTBVCRPQGGK (SEQ ID NO: 8);
GGTYSCHFGPLTBVCRPQ (SEQ ID NO: 9);
GTYSCHFGPLTBVCRPQ (SEQ ID NO: 10); and
GGTYSCHFGPLTBVCJPQGG (SEQ ID NO: 11).

15 Other preferred R¹ and R² sequences include, but are not limited to, the following:

20 GGTYSCHFGPLTBVCQPLGG (SEQ ID NO: 12);
GGLYACHJGPJTBVCQPLRG (SEQ ID NO: 13);
GGLYACHMGPMTBVCQPLRG (SEQ ID NO: 14);
GGTYSCHFGPLTBVCRPQGG (SEQ ID NO: 15);
GGLYLCRFGPVTBDCGYKGG (SEQ ID NO: 16);
VGNYMCHFGPITBVRPGGG (SEQ ID NO: 17);
GGVYACRMGPITBVCSPPLGG (SEQ ID NO: 18);
GGPHHVVACRMGPLTBIC (SEQ ID NO: 19);
25 TIAQYICYMGPETBECRSPSKA (SEQ ID NO: 20); and
YCHFGPLTBVC (SEQ ID NO: 21).

Numerous other sequences are also possible, however, including the following:

30 YSCHFGPLTBVCK (SEQ ID NO: 22);
GGTYSCHFGPLTBVCKPQ (SEQ ID NO: 23)
GGDYHCRMGPLTBVCKPLGG (SEQ ID NO: 24)

GGTYSCHFGPLTBVCKPQGG (SEQ ID NO: 25)
GGTYSCHFGPLTUVCRPQGG (SEQ ID NO: 26)
GGTYRCSMGPMTBVCLPMGG (SEQ ID NO: 27);
GGMYSCRMGPMTBVCGPSGG (SEQ ID NO: 28);
5 GGWAWCRMGPITBVCSAHGG (SEQ ID NO: 29);
GGMYSCRMGPMTBVCIPIYG (SEQ ID NO: 30);
GGEYKCYMGPITBVCKPEGG (SEQ ID NO: 31);
GGDYTCRMGPMTBICATGG (SEQ ID NO: 32);
GGNYLCRFGPGTBDCGFRG (SEQ ID NO: 33);
10 GGNYVCRMGPITBICTPAGG (SEQ ID NO: 34);
GGKDVCRMGPITBDCRSTGG (SEQ ID NO: 35);
GGSYLCRMGPPTBLCTAQRGGGN (SEQ ID NO: 36);
GGNYLCRMGPATBVCGRMGG (SEQ ID NO: 37);
GGEYKCRMGPPLTBVCQYAGG (SEQ ID NO: 38);
15 GGDYTCRMGPMTBICATRG (SEQ ID NO: 39);
GGVYVCRMGPPLTBECTASGG (SEQ ID NO: 40);
GGEYSCRMGPMTBVCSPPTGG (SEQ ID NO: 41);
GGEYLCRMGPITBVCERYGG (SEQ ID NO: 42);
GGNYICRMGPMTBVCTAHGG (SEQ ID NO: 43);
20 GGDYLCRMGPATBVCGRMGG (SEQ ID NO: 44);
GGLYSCRMGPITBVCTKAGG (SEQ ID NO: 45);
GGGYHCRMGPMTBVCRPVGG (SEQ ID NO: 46);
GGIYKCLMGPPLTBVCTPDGG (SEQ ID NO: 47);
GGLYSCLMGPITBLCKPKGG (SEQ ID NO: 48);
25 GGDYSCRMGPPTBVCTPPGG (SEQ ID NO: 49);
GGDYWCRMGPSTBECNAHGG (SEQ ID NO: 50);
GGKYLCSFGPITBVCARYGG (SEQ ID NO: 51);
GGLYKCRLGPITBVCSPPLGG (SEQ ID NO: 52);
GGSYTCRFGPETBVCRPNGG (SEQ ID NO: 53);
30 GGSYSCRMGPITBVKPGGG (SEQ ID NO: 54);
GGSYTCRMGPITBVCLPAGG (SEQ ID NO: 55);
GGLYECRMGPMTBVRPGGG (SEQ ID NO: 56);

-15-

GGDYTCRMGPITBICTKAGG (SEQ ID NO: 57);
GGVYCRMGPITBECNR YVG (SEQ ID NO: 58);
GGAYLCHMGPITBVCRPQGG (SEQ ID NO: 59);
GGEYSCRMGPNTBVCKPVGG (SEQ ID NO: 60);
5 GGLYLCRMGPVTBECQPRGG (SEQ ID NO: 61);
GGLYTCRMGPITBVCLLPGG (SEQ ID NO: 62);
GGLYTCRMGPVTBVCTGAGG (SEQ ID NO: 63);
GGVYKCRMGPPLTBECRPTGG (SEQ ID NO: 64);
GGDYNCRFGPLTBVCKPSGG (SEQ ID NO: 65);
10 GGSYLCRGPTTBLCSSAGG (SEQ ID NO: 66);
GGSYLCRMGPPTBVCTRMMGG (SEQ ID NO: 67);
GGSYLCRGPTTBLCTQRGG (SEQ ID NO: 68);
GGWVTCRMGPITBVCGVHGG (SEQ ID NO: 69);
GGQLLCGIGPITBVCRWVGG (SEQ ID NO: 70);
15 GGKYSCFMGPPTBVCSPVGRGV (SEQ ID NO: 71);
GGWVYCRIGPITBVCDTNGG (SEQ ID NO: 72);
GGMYYCRMGPMTBVCKGAGG (SEQ ID NO: 73);
GGTTQCWIGPITBVCRARGG (SEQ ID NO: 74);
GGPYHCRMGPITBVCGPVGG (SEQ ID NO: 75);
20 GGEYRCRMGPISBVCSPQGG (SEQ ID NO: 76);
GGNYTCRGPLTBECTPQGGGA (SEQ ID NO: 77);
GGSWDCRIGPITBVCKWSGG (SEQ ID NO: 78);
GGLYLCRMGPQTBMCPQPGGG (SEQ ID NO: 79);
GGDYVCRMGPMTBVCAPYGR (SEQ ID NO: 80);
25 GGWYSCLMGPMTBVCKAHRG (SEQ ID NO: 81);
GGKYYCWMGPMTBVCSAGG (SEQ ID NO: 82);
GGYVMCRIGPITBVCVIPGG (SEQ ID NO: 83);
GSCLQCCIGPITBVCRHAGG (SEQ ID NO: 84);
GGNYFCRMGPITBVCQRSVG (SEQ ID NO: 85);
30 GGEYICRMGPPLBECKRTGG (SEQ ID NO: 86);
GGLYACRMGPITBVCKYMAG (SEQ ID NO: 87);
GGQYLCTFGPITBLCRGAGG (SEQ ID NO: 88);

GGYTCRMPITBVCSAHGG (SEQ ID NO: 89);
GGTYKCWMGPMTBVCRPVGG (SEQ ID NO: 90);
GGNYYCRFGPITFECHPTGG (SEQ ID NO: 91);
GGEYLCRMPMTBVCTPVGG (SEQ ID NO: 92); and
5 GGLYTCAVGPITBVCLPAGG (SEQ ID NO: 93).

III. DIMER SYNTHESIS:

The compounds of the invention can be prepared in one of two ways. Both methods involve, initially, providing a linking molecule that may be any suitable compound having two functional groups capable of serving as initiation sites for peptide synthesis, and a third functional group attachable to a solid support. The linking molecule is typically a diamine having the structure $H_2N-R^3-NH_2$, where R^3 is lower (C_{1-6}) alkylene substituted with a functional group such as a carboxyl group that enables binding to another molecular moiety (e.g., as may be present on the surface of a solid support), and is optionally substituted with a lower alkyl group. Dimer synthesis can then proceed by: (A) first synthesizing peptide chain R^1 , with the two cysteine or homocysteine residues at X_3 and X_9 , protected with a first thiol protecting group, e.g., trityl (Trt), allyloxycarbonyl (Alloc), 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde), or the like, then synthesizing the second peptide chain R^2 , with the two cysteine or homocysteine residues at X'_3 and X'_9 , protected with a second thiol protecting group, different from the first, e.g., acetamidomethyl (Acm), t-butyl (tBu), or the like, then removing the first protecting group and effecting cyclization via a disulfide bond within R^1 , and, finally, removing the second protecting group and effecting cyclization via a disulfide bond within R^2 ; or (B) simultaneously synthesizing peptide chains R^1 and R^2 , using a single protecting group for 20 the four cysteine or homocysteine residues at X_3 , X_9 , X'_3 and X'_9 , removing the protecting group, and conducting an oxidative folding step which preferentially results in a dimer having an intramolecular disulfide bond linking X_3 and X_9 , in R^1 , and a second intramolecular disulfide bond linking X'_3 and X'_9 , in R^2 . These two methods are described in further detail below.

A. SEQUENTIAL SYNTHESIS OF R¹ AND R² AND REGIOSELECTIVE OXIDATION:

In one method, then, the initial linking molecule is protected with two different, orthogonally removable amine protecting groups. The protected diamine may be, for example, a protected 5 lysine molecule. The protected linking molecule is coupled to a solid support through a functional group, e.g., a carboxylic acid moiety as is present in lysine or an amide moiety as is present in lysine amide. One of the two amine protecting groups is removed, and the first peptide R¹ is synthesized using the unprotected amine moiety as a starting point. The cysteine or homocysteine residues present at X₃ and X₉ are protected with a thiol protecting group, 10 which is introduced by incorporation of the corresponding thiol protected amino acid during synthesis of the peptide chain. The remaining amine protecting group on the support-bound linking molecule is then removed, and the second peptide R² is synthesized using that unprotected amine moiety as a starting point. As with R¹, the cysteine or homocysteine residues present at X'₃ and X'₉ are protected with a thiol protecting group.

15 Standard solid phase peptide synthesis techniques are preferred for synthesis of R¹ and R², as described, for example, by Merrifield (1963) *J. Am. Chem. Soc.* 85:2149. As is well known in the art, solid phase synthesis using the Merrifield method involves successive coupling of α -amino protected amino acids to a growing support-bound peptide chain. After the initial coupling of a protected amino acid to a resin support (e.g., a polystyrene resin, a 20 chloromethylated resin, a hydroxymethyl resin, a benzhydrylamine resin, or the like, depending on the chemistry used), the α -amino protecting group is removed by a choice of reagents, depending on the specific protecting group. Suitable α -amino protecting groups are those known to be useful in the art of stepwise synthesis of peptides. Included are acyl type protecting groups (e.g., formyl, trifluoroacetyl, acetyl), aromatic urethane type protecting groups 25 (e.g., benzyloxycarbonyl (Cbz) and substituted Cbz), aliphatic urethane protecting groups (e.g., t-butyloxycarbonyl (Boc), isopropylloxycarbonyl, cyclohexyloxycarbonyl), alkyl type protecting groups (e.g., benzyl, triphenylmethyl), fluorenylmethyl oxycarbonyl (Fmoc), Alloc and Dde. The side chain protecting groups (typically ethers, esters, trityl, and the like) remain intact 30 during coupling; however, the side chain protecting group must be removable upon completion of the synthesis of the final peptide. Preferred side chain protecting groups, as will be appreciated by those skilled in the art, will depend on the particular amino acid that is being protected as well as the overall chemistry used. After removal of the α -amino protecting group, the

remaining protected amino acids are coupled stepwise in the desired order. Each protected amino acid is generally reacted in about a 3-fold excess using an appropriate carboxyl group activator such as 2-(1H-benzotriazol-1-yl)-1,1,3,3 tetramethyluronium hexafluorophosphate (HBTU) or dicyclohexylcarbodiimide (DCC) in solution, for example, in methylene chloride (CH₂Cl₂), N-methyl pyrrolidone, dimethyl formamide (DMF), or mixtures thereof.

To protect cysteine and homocysteine residues during synthesis of the peptide chains R¹ and R², protecting groups are covalently linked to the free thiol moieties during synthesis, as noted above. Orthogonal protection, where different protecting groups are used to protect thiol moieties on the different dimer chains, allows cyclization within a chain without cross-reactions between chains. This is preferable, insofar as dimers containing disulfide bonds linking R¹ and R² are substantially less active than dimers in which each of R¹ and R² contain a cyclic group linking the two cysteine or homocysteine residues at X₃ and X₉ and at X'₃ and X'₉, respectively. Following synthesis of R¹ and R², then, the first thiol protecting group (e.g., protecting the two cysteine or homocysteine residues in R¹) is removed, and intramolecular cyclization is effected within R¹ using an oxidizing reagent suitable for forming disulfide bonds from free thiols. This step, accordingly, results in covalent binding of X₃ to X₉ through a disulfide bond. The second thiol protecting group is then removed (protecting the two cysteine or homocysteine residues in R²), and intramolecular cyclization is effected in the second peptide chain, R², a similar manner. In this way, the dimer that is synthesized has a cyclic moiety in each of the two peptide chains, and disulfide bridges between the two peptide chains are not formed. As noted earlier herein, dimers in which each peptide chain contains a cyclic group are far more active than dimers in which the peptide chains are bridged through disulfide linkages, and it is thus highly preferable to optimize synthesis of the former dimer types.

After dimer synthesis is complete, the compound is cleaved from the solid support by treatment with a reagent such as trifluoroacetic acid, preferably in combination with a scavenger such as ethanedithiol, β -mercaptoethanol or thioanisole. The cleavage reagent not only cleaves the peptide from the resin, but also cleaves all remaining side chain protecting groups.

The above-described synthesis is a "regioselective" oxidation process in which orthogonally removable protecting groups are used for both amine protection of the support-bound linker and thiol protection within the newly synthesized peptide chains.

The synthesis is illustrated schematically in Figure 2. In the figure, the linking moiety L_K is an initial support-bound lysine residue. The lysine residue is shown as having a first

amine protecting group, Alloc, protecting the ϵ -amino moiety, and a second protecting group, Fmoc, bound through a β -alanine residue ("BA") to the α -amino moiety. Other amine protecting moieties may of course be used, and as noted earlier herein the β -alanine residues are optional.

The initial step of the synthesis involves removal of the Fmoc group using a suitable reagent,

5 usually mild base (e.g., 20% piperidine in DMF), and the free amine group that results is then used as the starting point for the synthesis of a first peptide chain. The N-terminus of the completed chain is shown protected with Boc. The first peptide chain contains two thiol residues at X_3 and X_9 , which are protected with a first protecting group P^1 ; these protecting groups are introduced during peptide synthesis by coupling thiol protected amino acids, i.e.,

10 cysteine or homocysteine in which the thiol moieties are protected with P^1 . The second amine protecting group, the Alloc moiety, is then removed with a suitable reagent (e.g., Pd(PPh_3)/4-methyl morpholine and chloroform), and the free amine group that results is then used as the starting point for the synthesis of a second peptide chain. Like the first peptide chain, the second peptide chain contains two thiol residues, one at X'_3 and the other at X'_9 . As may be

15 seen in the figure, the two thiol residues in the second peptide chain are protected with a second protecting group P^2 (and, as for the first peptide chain, these protecting groups are introduced during synthesis of the peptide chain). The first thiol protecting group P^1 is removed, and, simultaneously, the terminal amine protecting group (Boc) is removed, side chain protecting groups are removed, and the dimer is cleaved from the support. Intramolecular cyclization is

20 then effected within the first peptide chain using a suitable oxidizing reagent, e.g., aqueous dimethyl sulfoxide (DMSO). The second thiol protecting group, P^2 , is then removed, and intramolecular cyclization is effected within the second peptide chain, again using a suitable oxidizing reagent. Optimally, removal of the second thiol protecting group and intramolecular cyclization in the second peptide chain are effected simultaneously using, for example,

25 iodine/methanol (preferably in combination with thallium trifluoroacetate), trifluoroacetic acid/trimethylsilane, or the like.

B. SIMULTANEOUS SYNTHESIS OF R^1 AND R^2 AND OXIDATIVE FOLDING:

A much preferred method involves an oxidative folding technique wherein

30 orthogonally removable protecting groups are unnecessary and the overall number of reaction steps is reduced. In addition, when R^1 and R^2 are identical, as is the case in the preferred dimer compounds herein, the two peptide chains can be synthesized simultaneously.

In this method, then, the linking molecule is coupled to the solid support, as above, and synthesis of the two peptide chains of the dimer is carried out simultaneously using, for example, the Merrifield method as summarized in the preceding section. Protection of thiol groups in cysteine and homocysteine residues using two different protecting groups, as required

5 in the synthesis described in Section A, is unnecessary. After synthesis of R^1 and R^2 is complete, the dimer--having free cysteine and/or homocysteine moieties in each peptide chain, at X_3 , X_9 , X'_3 and X'_9 --is treated with an oxidizing composition effective to bring about intramolecular cyclization within each of the two peptide chains R^1 and R^2 while minimizing the formation of disulfide bridges therebetween. Generally, the oxidizing composition is an
10 aqueous solution of an oxidizing reagent that is of a type and present at a concentration effective to optimize formation of the desired isomer, wherein each peptide chain contains a cyclic group formed by covalent bonding of X_3 to X_9 , and X'_3 to X'_9 , through a disulfide linkage. A particularly preferred oxidizing reagent is dimethyl sulfoxide, preferably in water at a concentration of about 15% to 100% (v/v), more preferably in the range of about 50% to 100%
15 (v/v), and still more preferably in the range of 80% to 100% (v/v); most preferably the oxidizing composition comprises 100% DMSO. The cyclization reaction is conducted most advantageously at a temperature in the range of 5°C to 30°C for at least about 30 minutes, preferably at least about several hours, most preferably at least about 24 hours, and optimally for about 2 days.

20 This synthetic method is illustrated schematically in Figure 3. In Figure 3, the initial support-bound lysine residue is shown as having two identical amine protecting groups (Fmoc groups in the figure, for purposes of illustration) protecting the α - and ϵ -amino moieties. The Fmoc groups are removed with a suitable reagent, and two free amine groups result. The two peptide chains are synthesized at each of the two free amine groups, simultaneously, using a
25 single thiol protecting group P^1 for the cysteine and/or homocysteine residues. Following synthesis of the peptide chains, the dimer is cleaved from the solid support and subjected to oxidation using the oxidative folding technique just described. The desired isomer, in which a cyclic group is present in each of the two peptide chains (a disulfide bond links X_3 and X_9 in the first peptide chain, and a second disulfide bond links X'_3 and X'_9 in the second peptide chain),
30 predominates.

In Figure 4, the three isomers that can result from a one-step oxidative folding reaction are illustrated. Isomer 1 is far preferred, insofar as it is a substantially more active EPO

agonist than either Isomer 2 or Isomer 3 (see Figure 5, illustrating in graph form the comparative results vis-à-vis the individual isomers, as explained in Example 14). The aforementioned oxidative folding reaction preferentially gives rise to the desired compound, Isomer 1.

5

C. DIMER SYNTHESIS--VARIATIONS AND FURTHER MODIFICATIONS:

The procedures of parts (A) and (B) can also be used to synthesize dimers containing amino acids other than the 20 naturally occurring, genetically encoded amino acids. For instance, naphthylalanine can be substituted for tryptophan, with 1-naphthylalanine particularly preferred at X₇ and X'₇, facilitating synthesis and significantly improving activity. Other synthetic amino acids that can be substituted into the peptides of the present invention include, but are not limited to, nor-leucine and 3-pyridylalanine.

One can also modify the amino termini of the peptide chains of the dimer to produce other compounds of the invention. Amino terminus modifications include methylation (i.e., conversion of a free amino group to an -NHCH₃ or -N(CH₃)₂ moiety), acetylation (with either acetic acid *per se*, or with a halogenated derivative thereof such as α -chloroacetic acid, α -bromoacetic acid, or α -iodoacetic acid), addition of a benzyloxycarbonyl group, or blocking with a blocking group containing a carboxylate functionality RCOO- or a sulfonyl functionality R-SO₂-, where R is selected from the group consisting of alkyl, aryl, heteroaryl, alkyl aryl, and the like, and similar groups. One can also incorporate a desamino residue at the N-terminus, so that there is no terminal amino group, to decrease susceptibility to proteases or to restrict the conformation of the peptide chain or chains. Particularly preferred modifications herein, as explained in Section (II), are wherein the amino termini of both R¹ and R² are acetylated or conjugated to a polyethylene glycol molecule, with the latter modification most preferred.

When N-terminal modification is desired, it is also preferred that the peptide chains contain no amino groups other than at the N-termini (and thus no lysine residues), so that N-terminal-specific modification may be carried out.

One can also replace the naturally occurring side chains of the 20 genetically encoded amino acids (or the stereoisomeric D amino acids) with other side chains, for instance with groups such as alkyl, lower alkyl, cyclic 4-, 5-, 6-, to 7-membered alkyl, amide, amide lower alkyl, amide di(lower alkyl), lower alkoxy, hydroxy, carboxy and the lower ester derivatives thereof, and 4-, 5-, 6-, to 7-membered heterocyclic. In particular, proline analogues in which the

ring size of the proline residue is changed from 5 members to 4, 6, or 7 members can be employed.

One can also readily modify the peptide dimers herein by phosphorylation or other methods as described in Hruby et al., 1 Jun. 1990, *Biochem J.* 268:249-262. Thus, the peptide 5 dimers of the invention also serve as structural models for non-peptidic compounds with similar biological activity. For example, the peptide backbones may be replaced with a backbone composed of phosphonates, amides, carbamates, sulfonamides, secondary amines, and N-methylamino acids.

10

IV. UTILITY

The compounds of the invention are useful *in vitro* as unique tools for understanding the biological role of EPO, including the evaluation of the many factors thought to influence, and be influenced by, the production of EPO and the binding of EPO to the EPO-R (e.g., the 15 mechanism of EPO signal transduction/receptor activation). The present compounds are also useful in the development of other compounds that bind to the EPO-R, because the present compounds provide important structure-activity relationship (SAR) information that facilitate that development.

Moreover, based on their ability to bind to the EPO receptor, the compounds of the 20 present invention can be used as reagents for detecting EPO receptors on living cells, fixed cells, in biological fluids, in tissue homogenates, in purified, natural biological materials, etc. For example, by labeling such peptides, one can identify cells having EPO-R on their surfaces. In addition, based on their ability to bind the EPO receptor, the peptide dimers of the present invention can be used in *in situ* staining, FACS (fluorescence-activated cell sorting), Western 25 blotting, ELISA (enzyme-linked immunoabsorptive assay), etc. In addition, based on their ability to bind to the EPO receptor, the peptide dimers of the present invention can be used in receptor purification or in purifying cells expressing EPO receptors on the cell surface (or inside permeabilized cells).

The compounds of the invention can also be utilized as commercial research reagents 30 for various medical research and diagnostic uses. Such uses can include but are not limited to: (1) use as a calibration standard for quantitating the activities of candidate EPO agonists in a variety of functional assays; (2) use as blocking reagents in random peptide screening, i.e., in

looking for new families of EPO receptor peptide ligands, the peptides can be used to block recovery of the presently claimed EPO peptides; (3) use in the co-crystallization with EPO receptor, i.e., the peptide dimers of the present invention will allow formation of crystals bound to the EPO receptor, enabling the determination of receptor/peptide structure x-ray

5 crystallography; (4) use to measure the capacity of erythrocyte precursor cells to differentiate and thus stimulate the induction of globin synthesis and increases in the synthesis of the heme complex and in the number of ferritin receptors; (5) use to maintain the proliferation and growth of EPO-dependent cell lines, such as the FDCP-1-mEPO-R and the TF-1 cell lines; and (6) other research and diagnostic applications wherein the EPO-receptor is preferably activated 10 or such activation is conveniently calibrated against a known quantity of an EPO agonist, and the like.

The compounds of the invention can also be administered to warm blooded animals, including humans, to simulate the binding of EPO to the EPO-R *in vivo*. Thus, the present invention encompasses methods for therapeutic treatment of disorders associated with a 15 deficiency of EPO that comprise administering a compound of the invention in amounts sufficient to stimulate the EPO-R and thus alleviate the symptoms associated with a deficiency of EPO *in vivo*. For example, the compounds of this invention will find use in the treatment of end-stage renal failure/dialysis; anemia associated with AIDS, anemia associated with chronic inflammatory diseases (for example, rheumatoid arthritis and chronic bowel inflammation), 20 autoimmune disease, and malignancies; and for boosting the red blood count of a patient prior to surgery.

Other embodiments of this invention provide for the administration of the compounds of the invention for the treatment of disorders which are not characterized by low or deficient red blood cells, for example as a pretreatment prior to transfusions. In addition, administration 25 of the compounds of this invention can result in a decrease in bleeding time and thus will find use in the administration to patients prior to surgery or for indications wherein bleeding is expected to occur. In addition, the compounds of this invention will find use in the activation of megakaryocytes.

Since EPO has been shown to have a mitogenic and chemotactic effect on vascular 30 endothelial cells as well as an effect on central cholinergic neurons (see, e.g., Amagnostou et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:5978-5982 and Konishi et al. (1993) *Brain Res.* 609:29-35), the compounds of this invention will also find use for the treatment of a variety of

vascular disorders, such as promoting wound healing, growth of collateral coronary blood vessels (such as those that may occur after myocardial infarction), trauma, and post-vascular graft treatment, and a variety of neurological disorders, generally characterized by low absolute levels of acetyl choline or low relative levels of acetyl choline as compared to other neuroactive substances e.g., neurotransmitters.

Accordingly, the present invention includes pharmaceutical compositions comprising, as an active ingredient, at least one of the peptide dimers of the invention in association with a pharmaceutical carrier or diluent. The compounds of this invention can be administered by oral, parenteral (intramuscular, intraperitoneal, intravenous (IV) or subcutaneous) injection, transdermal (either passively or using iontophoresis or electroporation), or transmucosal (nasal, vaginal, rectal, or sublingual) routes of administration, or using bioerodible inserts, and can be formulated in dosage forms appropriate for each route of administration.

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound is admixed with at least one inert pharmaceutically acceptable carrier such as sucrose, lactose, or starch. Such dosage forms can also comprise, as is normal practice, additional substances other than inert diluents, e.g., lubricating, agents such as magnesium stearate. In the case of capsules, tablets, and pills, the dosage forms may also comprise buffering agents. Tablets and pills can additionally be prepared with enteric coatings.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, with the elixirs containing inert diluents commonly used in the art, such as water. Besides such inert diluents, compositions can also include adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring, and perfuming agents.

Preparations according to this invention for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, or emulsions. Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil and corn oil, gelatin, and injectable organic esters such as ethyl oleate. Such dosage forms may also contain adjuvants such as preserving, wetting, emulsifying, and dispersing agents. They may be sterilized by, for example, filtration through a bacteria retaining filter, by incorporating sterilizing agents into the compositions, by irradiating the compositions, or by heating the

compositions. They can also be manufactured using sterile water, or some other sterile injectable medium, immediately before use.

Compositions for rectal or vaginal administration are preferably suppositories which may contain, in addition to the active substance, excipients such as cocoa butter or a suppository wax. Compositions for nasal or sublingual administration are also prepared with standard excipients well known in the art.

The dosage of active ingredient in the compositions of this invention may be varied; however, it is necessary that the amount of the active ingredient shall be such that a suitable dosage form is obtained. The selected dosage depends upon the desired therapeutic effect, on the route of administration, and on the duration of the treatment desired. Generally, dosage levels of between 0.001 to 10 mg/kg of body weight daily are administered to mammals.

It is to be understood that while the invention has been described in conjunction with the preferred specific embodiments thereof, that the foregoing description as well as the examples which follow are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

All patents, patent applications, and publications mentioned herein are hereby incorporated by reference in their entirety.

20

EXPERIMENTAL

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to prepare and use the compounds disclosed and claimed herein. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.) but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C and pressure is at or near atmospheric.

The resin used (PAL) was obtained commercially available from Milligen/Bioscience. Solid phase reactions were carried out at room temperature. Unless otherwise indicated, all starting materials and reagents were obtained commercially, e.g., from Aldrich, Sigma and ICN, and used without further purification.

Also, in these examples and throughout this specification, the abbreviations employed have their generally accepted meanings, as follows:

5 Acm = acetamidomethyl

Alloc = allyloxycarbonyl

Boc = *t*-butoxycarbonyl

Bz = benzyl

Cbz = benzyloxycarbonyl

10 DMF = dimethyl formamide

Fmoc = fluorenylmethyl oxycarbonyl

15 g = gram

HBTU = O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HOEt = 1-hydroxybenztriazole

15 ml = milliliter

mmol = millimole

MMT = monomethoxytrityl

Mts = mesitoysulfonyl

20 Pmc = 2,2,5,7,8-pentamethylchroman sulfonate

tBu = *t*-butyl

Tos = tosyl

Trt = trityl

EXAMPLE 1

25 DIMER SYNTHESIS--SEQUENTIAL SYNTHESIS OF THE INDIVIDUAL PEPTIDE CHAINS:

A dimeric compound of the invention was synthesized, the compound comprising two identical peptide chains having the sequence GGTYSCHFGPLTBVCRPQGG (SEQ ID NO: 7), wherein each peptide chain is linked at its carboxyl terminus to a lysine (K) amide linking group, with the two cysteine residues in the first peptide chain linked to each other through a disulfide bond, and, similarly, the two cysteine residues in the second peptide chain linked to each other through a disulfide bond. Initially, Fmoc-Lys-Alloc was coupled to PAL resin (Milligen/Bioscience), a cross-linked polystyrene, thereby providing an initial lysine residue to serve as the linker between the two chains to be synthesized. The Fmoc protecting group was removed with mild base (20% piperidine in DMF). The first peptide chain was then synthesized using the resulting free amino group, the α -NH₂ group, as a starting point. Peptide synthesis was conducted using the Merrifield solid phase synthesis technique (see, Stewart, J. M., and Young, J. D., *Solid Phase Peptide Synthesis*, 2d. edition (Pierce Chemical, Rockford, Ill., 1984)) on an Applied Biosystems 433A automated instrument. Primary amine protection on amino acids was achieved with Fmoc, and side chain protecting groups were tBu for serine,

threosine and tyrosine, Trt for glutamine and asparagine, and Pmc for the arginine guanidino group. The two cysteine residues in the first peptide chain were protected with Trt. Each coupling was performed for either one or two hours with HBTU and HOBr.

Following synthesis of the first peptide chain, the Alloc group was removed from the support-bound lysine linker with Pd[P(C₆H₅)₃]₄, 4-methyl morpholine and chloroform to allow synthesis of the second peptide chain (to remove Dde, 2% NH₂-NH₂ in DMF may be used). The second peptide chain was then synthesized on the ε-NH₂ group using the techniques described above, with Trt again used to protect the two cysteine residues.

After synthesis of the second peptide chain was complete, the dimer was cleaved from the PAL support with a mixture of 90% trifluoroacetic acid, 5% ethanedithiol, and 5% water, initially at 4°C, and gradually increasing to room temperature over 1.5 hours; the trifluoroacetic acid also removed all trityl protecting groups in both of the peptide chains. The deprotected product was filtered from the resin and precipitated with diethyl ether. After thorough drying the product was purified by C18 reverse phase high performance liquid chromatography with a gradient of acetonitrile/water in 0.1% trifluoroacetic acid. The resulting dimer has four free thiol groups, with two in each peptide chain. The identity of the product was confirmed by ESMS. The final yield was approximately 5% based on the initial loading of the resin.

20

EXAMPLES 2-5

The procedures of Example 1 were repeated to provide a number of additional dimers, set forth in Table 1. In the table, L_K, R¹, R², n₁ and n₂ are defined according to structural formula (I)

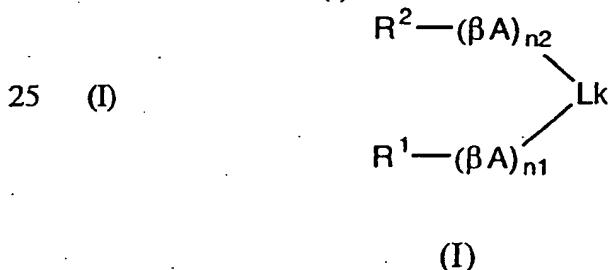


TABLE 1: PEPTIDE DIMERS WITH FREE CYSTEINE THIOLS

30

Example No.	SEQ ID NO:	R ¹ and R ²	n1	n2	Linker	Code No.

Example No.	SEQ ID NO:	R ¹ and R ²	n1	n2	Linker	Code No.
(1)	7	GGTYSCHFGPLTBVCRPQGG	0	0	lysine	17218
2	7	GGTYSCHFGPLTBVCRPQGG	1	0	lysine	20528
3	7	GGTYSCHFGPLTBVCRPQGG	1	1	lysine	20526
4	9	GGTYSCHFGPLTBVCRPQ	0	0	lysine	20532
5	10	GTYSCHFGPLTBVCRPQ	0	0	lysine	20531

EXAMPLE 6

REGIOSELECTIVE OXIDATION:

A peptide dimer was synthesized as in Example 1, except that (1) cysteine residues in the second peptide chain were protected with Acm rather than Trt, and (2) following removal of the Trt protecting groups, the Acm groups were allowed to remain in place to enable regioselective oxidation. That is, an initial disulfide linkage, in the first peptide chain, was formed by (1) removing the Trt protecting groups using trifluoroacetic acid, followed by (2) stirring in 20% DMSO/water overnight. The Acm protecting groups in the second peptide chain were then removed by treatment with iodine/methanol and thallium trifluoroacetate, which also oxidized the second peptide chain to form a second intramolecular cyclic group. The preferred isomer was obtained in high yield.

EXAMPLES 7-9

The procedures of Example 6 were repeated to provide a number of additional dimers, set forth in Table 2; like the compound prepared in Example 6, each of these dimers contained an intramolecular cyclic group in the first peptide chain (formed via a disulfide bridge between the two cysteine residues), and a second intramolecular cyclic group in the second peptide chain (again, formed via a disulfide bridge between the two cysteine residues). In the table, L_K, R¹, R², n1 and n2 are defined according to structural formula (I), above.

20

TABLE 2: PEPTIDE DIMERS WITH AN INTRAMOLECULAR CYCLIC GROUP IN EACH PEPTIDE CHAIN

Example No.	SEQ ID NO:	R ¹ and R ²	n1	n2	Linker	Code No.

Example No.	SEQ ID NO:	R ¹ and R ²	n1	n2	Linker	Code No.
(6)	7	GGTYSCHFGPLTBVCRPQGG	0	0	lysine	19957
7	7	GGTYSCHFGPLTBVCRPQGG	1	0	lysine	17286
8	7	GGTYSCHFGPLTBVCRPQGG	1	1	lysine	19261
9	11	GGTYSCHFGPLTBVCJPQGG	1	0	lysine	17393

EXAMPLES 10-12

AMINO TERMINUS MODIFICATION:

It will be appreciated by those skilled in the art that one can also modify the amino terminus of the first and/or second peptide chains within the compounds of the invention to produce additional compounds of the invention. Particularly preferred modifications involve (1) acetylation of the two amino termini of the dimer, and (2) "PEGylation" of the two amino termini of the dimer, i.e., covalent attachment of polyethylene glycol moieties.

The modified dimer compounds prepared are set forth in Table 3:

10

**TABLE 3: PEPTIDE DIMERS MODIFIED AT THE N-TERMINUS
OF EACH PEPTIDE CHAIN**

Example No.	SEQ ID NO:	R ¹ and R ² , end modifier	n1	n2	Linker	Code No.
10	7	GGTYSCHFGPLTBVCRPQGG, both N-termini acetylated	1	0	lysine	17644
11	7	GGTYSCHFGPLTBVCRPQGG, both N-termini PEGylated	1	1	lysine	17762
12	8	GGTYSCHFGPLTBVCRPQGGK both N-termini acetylated	0	0	=O	20952

15

EXAMPLE 13

OXIDATIVE FOLDING:

The peptide dimer of Example 6 was synthesized using an alternative technique in which the two peptide chains were synthesized simultaneously (in contrast to the successive synthesis set forth in Example 1) and intramolecular cyclization between cysteine residues in

each peptide chain was obtained by a single oxidative step (rather than by a multi-step regioselective process as described in Example 6).

Fmoc-Lys-Fmoc was coupled to PAL resin (Milligen/Bioscience), thereby providing an initial lysine residue to serve as the linker between the two chains to be synthesized. The 5 Fmoc protecting groups were removed with mild base (20% piperidine in DMF), and the peptide chains were then synthesized using the resulting free amino groups as starting points. As in Example 1, peptide synthesis was conducted using Merrifield solid phase synthesis on an Applied Biosystems 433A automated instrument. Primary amine protection on amino acids was achieved with Fmoc, and side chain protecting groups were t-butyl for serine, threonine and 10 tyrosine, trityl for glutamine and asparagine, and Pmc for the arginine guanidino group; Trt was used to protect all cysteine residues. After synthesis of the peptide, the dimer was cleaved from the PAL support with a mixture of 90% trifluoroacetic acid, 5% ethanedithiol, and 5% water, as in Example 1. The deprotected product was filtered from the resin and precipitated with diethyl ether. After thorough drying the product was purified by C18 reverse phase high performance 15 liquid chromatography with a gradient of acetonitrile/water in 0.1% trifluoroacetic acid.

Oxidation of the cysteine residues can produce three different isomers of the peptide dimer, as illustrated in Figure 4: (1) Isomer 1, wherein each peptide chain contains a cyclic group formed by a disulfide linkage between cysteine residues within the chain; (2) Isomer 2, containing disulfide linkages between analogous cysteine residues on opposite monomer chains; 20 and (3) Isomer 3, containing disulfide linkages between nonanalogous cysteines on opposite monomers. Isomer 1 is far preferred, insofar as it is a substantially more active EPO agonist than either Isomer 2 or Isomer 3.

Thus, to oxidize the peptide dimer and link the cysteine residues in the preferred manner, the dimer (having all cysteine thiols unprotected), was incubated in an aqueous solution 25 of DMSO (20% DMSO - 80% water, v/v) at 5°C, 1 mg/ml, for several hours. All three isomers were obtained, with approximately 50% of the product comprising Isomer 1. The process was then repeated using an aqueous solution of DMSO with an increased DMSO concentration (50% DMSO - 50% water, v/v). Again, all three isomers were obtained, with approximately 75% of the product comprising Isomer 1. The most preferred method, which yielded the largest 30 fraction of the desired isomer, employed 100% DMSO (at a temperature between 5°C and 25°C, with a reaction time of at least several hours, optimally 2 to 3 days).

EXAMPLE 14

BIOASSAY:

Peptide dimers of the invention were evaluated with respect to in vitro biological activity using an EPO receptor binding assay and a cell proliferation assay in which bioactivity was determined by Ba/F3-huEPOR cell proliferation. The protocol for each assay is described in Wrighton et al. (1997) *Nature Biotechnology* 15:1261-1265, and in U.S. Patent Nos. 5,773,569 and 5,830,851, both to Wrighton et al. and cited earlier herein. EC₅₀ values for the dimers prepared in Examples 1-12 are set forth in Table 4 (the EC₅₀ value is the concentration of compound required to produce 50% of the maximal activity obtained with recombinant erythropoietin).

TABLE 4: BIOASSAY RESULTS

Dimer-Example No.	Code	EC ₅₀ (nM)
1	17218	6
2	20528	6
3	20526	5
4	20532	50
5	20531	100
6	19957	0.5
7	17286	0.4
8	19261	0.3
9	17393	2
10	17644	5
11	17762	0.4

Dimer-Example No.	Code	EC ₅₀ (nM)
12	20952	2

The compounds of the invention are thus highly active EPO agonists. Their activity is significantly higher than the monomeric compounds; for example, a monomeric compound having the sequence GGTYSCHFGPLTBVCRPQGG (SEQ ID NO: 7), a compound previously viewed as a highly active EPO agonist (see U.S. Patent No. 5,773,569 to Wrighton et al.), was determined to have an EC₅₀ of 200 nM using the above-described assay. In addition, both N-terminal PEGylation and replacement of lysine (K) with 1-naphthylalanine at X₇ and X'₇ were found to significantly increase potency.

With regard to the different isomers of the dimer prepared in Example 13 (see Figure 4), i.e., Isomer 1 (Code No. 19957), Isomer 2 (Code No. 19954) and Isomer 3 (Code No. 19965), Figure 5 illustrates that the in vitro bioactivity of Isomer 1 was noticeably higher than the measured in vitro bioactivity of any of the other compounds.

WHAT IS CLAIMED IS:

1. A method for synthesizing a peptide dimer, comprising:

(a) providing a linking moiety L_K having first and second functional groups capable

5 of serving as initiation sites for peptide synthesis, and a third functional group attachable to a solid support;

(b) binding the linking moiety L_K to a solid support through the third functional group;

(c) synthesizing a first peptide segment at the first functional group and a second

10 peptide segment at the second functional group, wherein each of said first and second peptide segments contain two cysteine residues positioned to allow intramolecular cyclization through a disulfide bond;

(d) oxidizing the compound provided in step (c) in a manner effective to promote formation of disulfide bonds between cysteine residues in the same peptide segment while

15 minimizing formation of disulfide bonds between cysteine residues in different peptide segments.

2. The method of claim 1, wherein step (d) comprises treatment with an oxidizing

composition containing an oxidizing reagent of a type and in an amount effective to minimize

20 reaction products in which a cysteine residue of the first peptide segment binds to a cysteine residue of the second peptide segment.

3. The method of claim 2, wherein the oxidizing reagent is dimethyl sulfoxide.

25 4. The method of claim 3, wherein the oxidizing composition comprises approximately 15% to 100% (v/v) dimethyl sulfoxide.

5. The method of claim 4, wherein the oxidizing composition comprises approximately 50% to 100% (v/v) dimethyl sulfoxide.

30

6. The method of claim 5, wherein the oxidizing composition comprises approximately 50% to 100% (v/v) dimethyl sulfoxide.

7. The method of claim 6, wherein the oxidizing composition comprises approximately 80% to 100% (v/v) dimethyl sulfoxide.

8. The method of claim 7, wherein the oxidizing composition comprises 5 approximately 100% (v/v) dimethyl sulfoxide.

9. The method of claim 1, wherein:

the first peptide segment is approximately 10 to 40 amino acid residues in length, binds to the erythropoietin receptor, and contains a sequence of amino acids

10 $X_3X_4X_5GPX_6TX_7X_8X_9$ (SEQ ID NO: 1) wherein each amino acid is indicated by standard one-letter abbreviation, X_3 is C or homocysteine (Hoc), X_4 is R, H, L or W, X_5 is M, F, I or nor-leucine (J), X_6 is selected from any one of the 20 genetically coded L-amino acids and J, X_7 is W, 1-naphthylalanine (B) or 2-naphthylalanine (U), X_8 is D, E, I, L or V, and X_9 is C or Hoc; and

15 the second peptide segment is approximately 10 to 40 amino acid residues in length, binds to the erythropoietin receptor, and contains a sequence of amino acids

$X'_3X'_4X'_5GPX'_6TX'_7X'_8X'_9$ (SEQ ID NO: 2) wherein each amino acid is indicated by standard one-letter abbreviation, X'_3 is C or Hoc, X'_4 is R, H, L or W, X'_5 is M, F, I or J, X'_6 is selected from any one of the 20 genetically coded L-amino acids and J, X'_7 is W, B or U, X'_8 is D, E, I, L 20 or V, and X'_9 is C or Hoc.

GGTYSCHFGPLTBVCRPQGG	SEQ ID NO: 7
GGTYSCHFGPLTBVCRPQGGK	SEQ ID NO: 8
GGTYSCHFGPLTBVCRPQ	SEQ ID NO: 9
GTYSCHFGPLTBVCRPQ	SEQ ID NO: 10
GGTYSCHFGPLTBVCJPQGG	SEQ ID NO: 11
GGTYSCHFGPLTBVCQPLGG	SEQ ID NO: 12
GGLYACHJGPJTBCQPLRG	SEQ ID NO: 13
GGLYACHMGPMTBVCQPLRG	SEQ ID NO: 14
GGTYSCHFGPLTBVCRPQGG	SEQ ID NO: 15
GGLYLCRFGPVTBDCGYKGG	SEQ ID NO: 16
VGNYMCHFGPITBVCRPGGG	SEQ ID NO: 17
GGVYACRMGPITBVCSPPLGG	SEQ ID NO: 18
GGPHHVYACRMGPLTBIC	SEQ ID NO: 19
TIAQYICYMGPETBECRSPPKA	SEQ ID NO: 20
YCHFGPLTBVC	SEQ ID NO: 21
YSCHFGPLTBVCK	SEQ ID NO: 22
GGTYSCHFGPLTBVCKPQ	SEQ ID NO: 23
GGDYHCRMGPITBVKPLGG	SEQ ID NO: 24
GGTYSCHFGPLTBVKPQGG	SEQ ID NO: 25
GGTYSCHFGPLTUVCRPQGG	SEQ ID NO: 26
GGTYRCRSMGPMTBVCLPMGG	SEQ ID NO: 27
GGMYSCRMPGPMTBVCGPSGG	SEQ ID NO: 28
GGWAWCRMGPITBVCSAHGG	SEQ ID NO: 29
GGMYSCRMPGPMTBVCIPYGG	SEQ ID NO: 30
GGEYKCYMGPITBVKPEGG	SEQ ID NO: 31
GGDYTCRMPGPMTBICTATGG	SEQ ID NO: 32
GGNYLCRFGPGTBDCFGFRG	SEQ ID NO: 33
GGNYVCRMGPITBICTPAGG	SEQ ID NO: 34
GGKDVCRMGPITBDCRSTGG	SEQ ID NO: 35
GGSYLCRMPGPTTBLCTAQRGGGN	SEQ ID NO: 36
GGNYLCRMPGPATBVCGRMGG	SEQ ID NO: 37
GGEYKCRMGPPLTBVCQYAGG	SEQ ID NO: 38
GGDYTCRMPGPMTBICTATRG	SEQ ID NO: 39
GGVYVCRMGPLTBECTASGG	SEQ ID NO: 40

Figure 1-1

GGEYSCRMGPMTBVCSPTGG	SEQ ID NO: 41
GGEYLCRMGPITBVCEERYGG	SEQ ID NO: 42
GGNYICRMGPMTBVCTAHGG	SEQ ID NO: 43
GGDYLCRMGPATBVCGRMGG	SEQ ID NO: 44
GGLYSCRMGPITBVCTKAGG	SEQ ID NO: 45
GGGYHCRMGPMTBVCRPVGG	SEQ ID NO: 46
GGIYKCLMGPPLTBVCTPDGG	SEQ ID NO: 47
GGLYSCLMGPITBLCKPKGG	SEQ ID NO: 48
GGDYSCRMGPPTTBVCTPPGG	SEQ ID NO: 49
GGDYWCRMGPSTBECNAHGG	SEQ ID NO: 50
GGKYLCSCFGPITBVCAHYGG	SEQ ID NO: 51
GGLYKCRLGPITBVCSPLGG	SEQ ID NO: 52
GGSYTCRFGPETBVCRPNNG	SEQ ID NO: 53
GGSYSCRMGPITBVCKPGGG	SEQ ID NO: 54
GGSYTCRMGPITBVCLPAGG	SEQ ID NO: 55
GGLYECRMGPMTBVCRPGGG	SEQ ID NO: 56
GGDYTCRMGPITBICKAGG	SEQ ID NO: 57
GGVYSCRMGPPTTBECNRYVG	SEQ ID NO: 58
GGAYLCHMGPITBVCRPQGG	SEQ ID NO: 59
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GGLYTCRMGPITBVCLLPGG	SEQ ID NO: 62
GGLYTCRMGPVTVCTGAGG	SEQ ID NO: 63
GGVYKCRMGPPLTBECRPTGG	SEQ ID NO: 64
GGDYNCRFGPLTBVCKPSGG	SEQ ID NO: 65
GGSYLCRFGPPTBLCSSAGG	SEQ ID NO: 66
GGSYLCRMGPPTBVCTRMMG	SEQ ID NO: 67
GGSYLCRFGPPTBLCTQRGG	SEQ ID NO: 68
GGWVTCRMGPITBVCGVHGG	SEQ ID NO: 69
GGQLLCGIGPITBVCRWVGG	SEQ ID NO: 70
GGKYSCFMGPTTBVCSPVGRGV	SEQ ID NO: 71
GGWVYCRIGPITBVCDTNGG	SEQ ID NO: 72
GGMYYCRMGPMTBVCKGAGG	SEQ ID NO: 73
GGTTQCWIGPITBVCRARGG	SEQ ID NO: 74

Figure 1-2

GGPYHCRMGPITBVCVPVGG	SEQ ID NO: 75
GGEYRCRMGPISBVCSPQGG	SEQ ID NO: 76
GGNYTCRFGPLTBECTPQGGGA	SEQ ID NO: 77
GGSWDCRIGPITBVCKWSGG	SEQ ID NO: 78
GGLYLCRMGPQTBMCPQPGGG	SEQ ID NO: 79
GGDYVCRMGPMTBVCAPYGR	SEQ ID NO: 80
GGWYSCLMGPMTBVCKAHRG	SEQ ID NO: 81
GGKYYCWMGPMTBVCSPAGG	SEQ ID NO: 82
GGYVMCRIGPITBVCDIPGG	SEQ ID NO: 83
GSCLQCCIGPITBVCRHAGG	SEQ ID NO: 84
GGNYFCRMGPITBVCQRSVG	SEQ ID NO: 85
GGEYICRMGPLTBECKRTGG	SEQ ID NO: 86
GGLYACRMGPITBVCKY MAG	SEQ ID NO: 87
GGQYLCTFGPITBLCRGAGG	SEQ ID NO: 88
GGYTTCRMGPITBVC SAHGG	SEQ ID NO: 89
GGTYKCWMGPMTBVC RPVGG	SEQ ID NO: 90
GGNYYCRFGPITFECHPTGG	SEQ ID NO: 91
GGEYLCRMGPMTBVCTPVGG	SEQ ID NO: 92
GGLYTCRMGPITBVCLPAGG	SEQ ID NO: 93

Figure 1-3

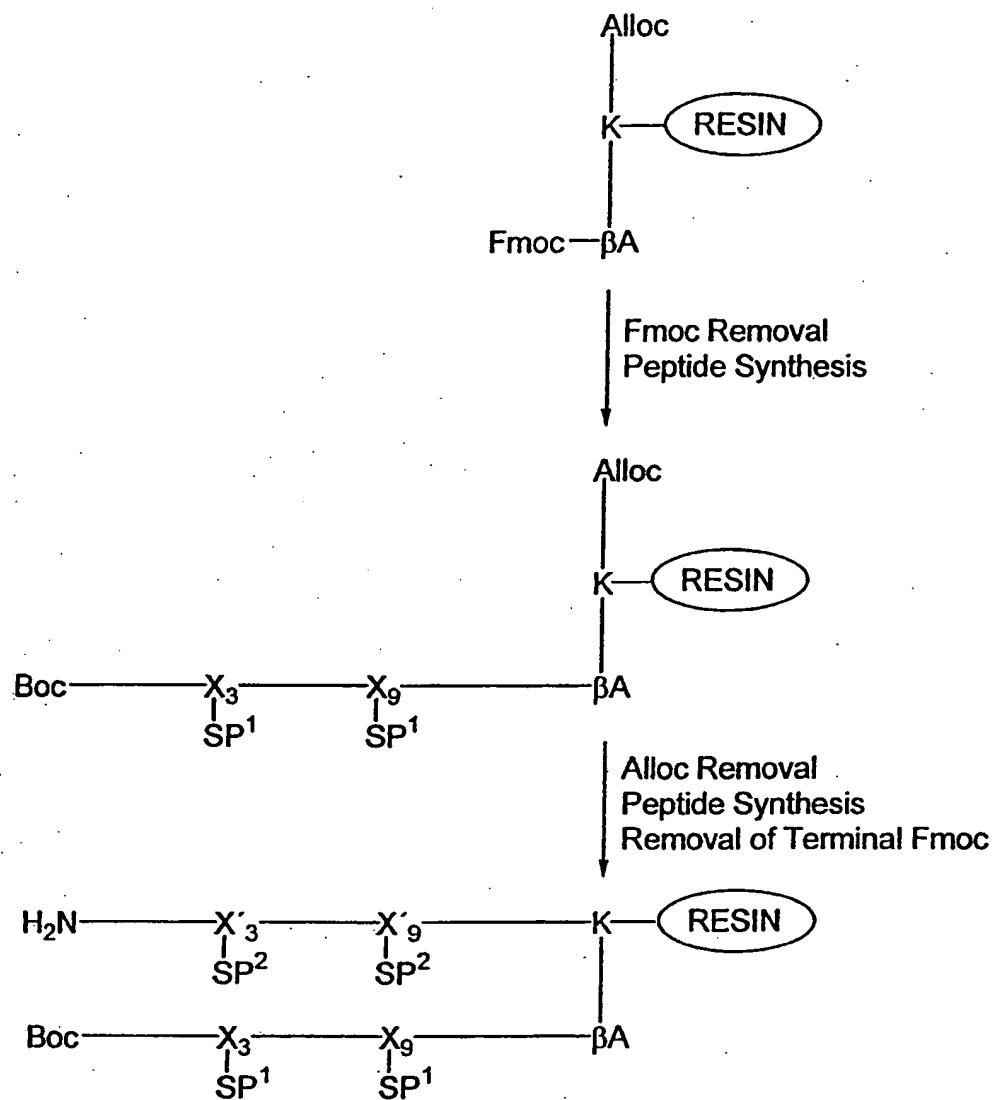


Figure 2-1

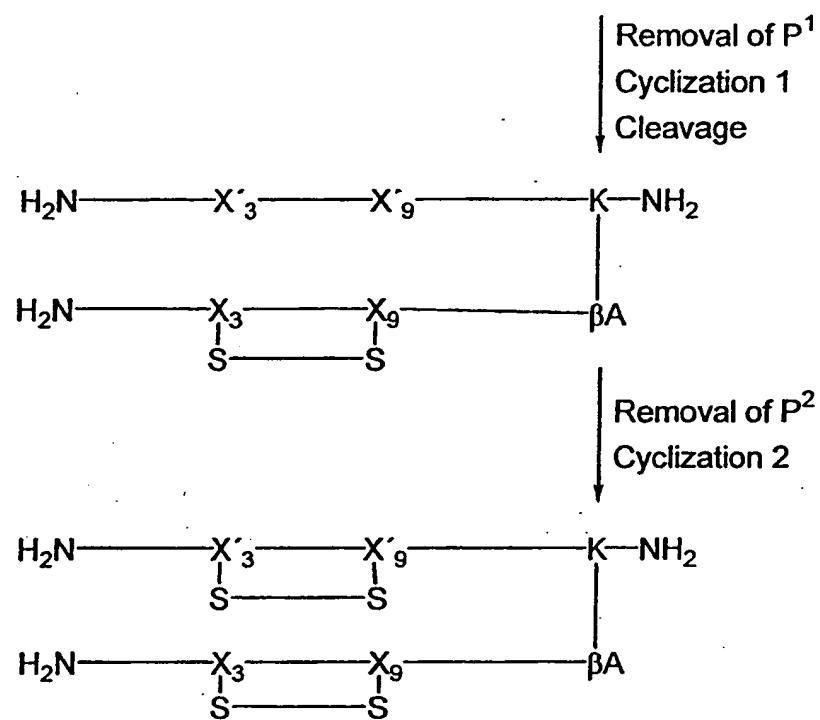


Figure 2-2

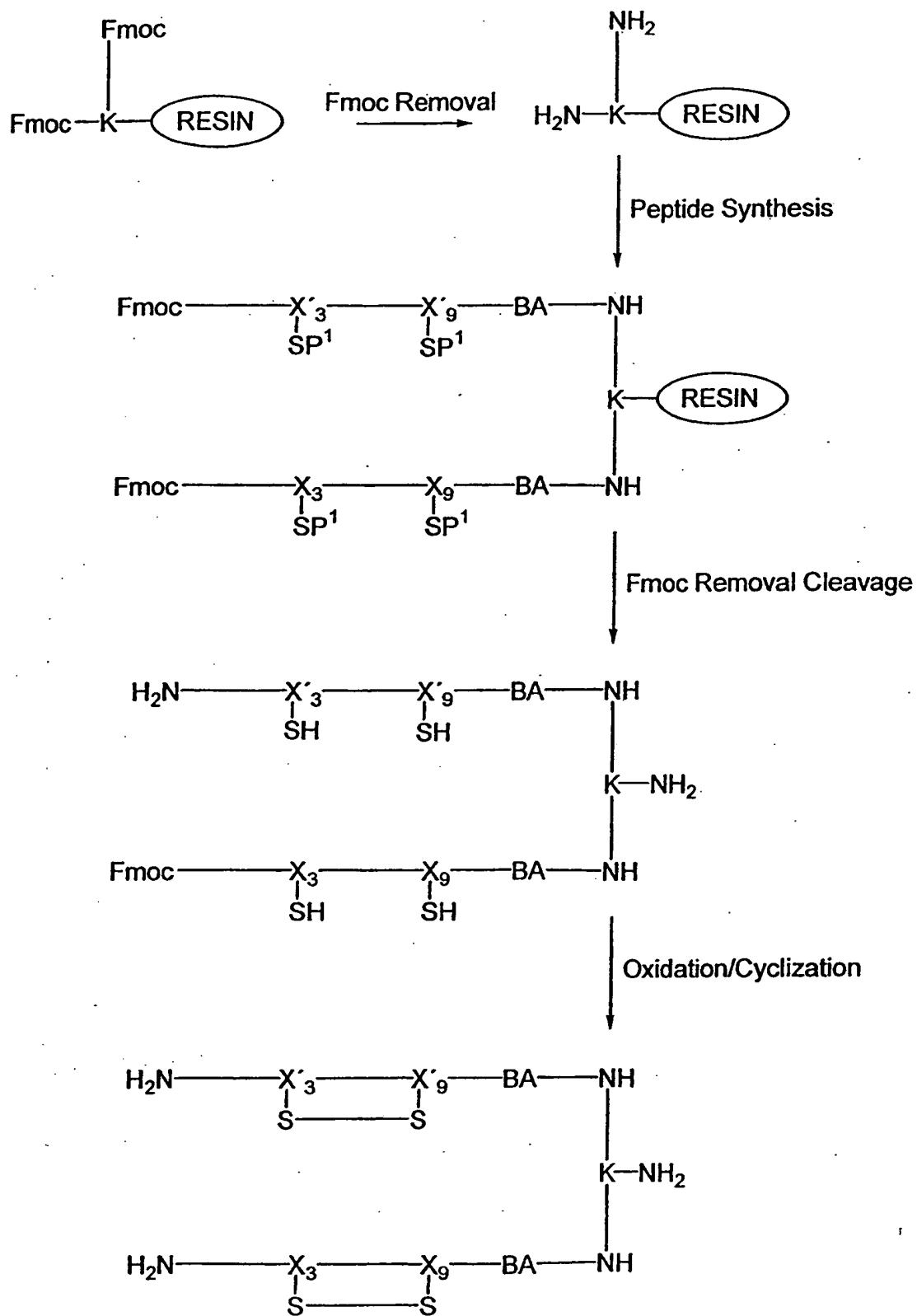


Figure 3

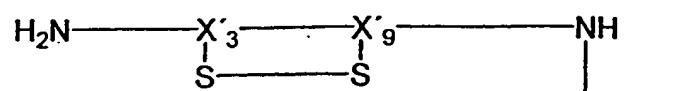
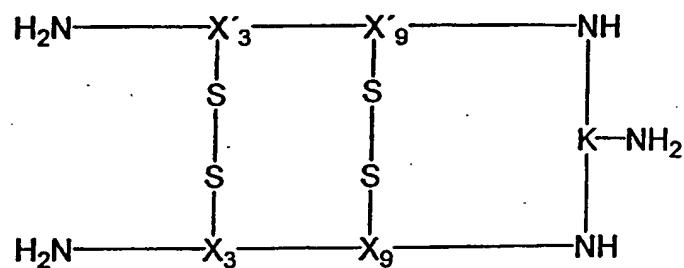
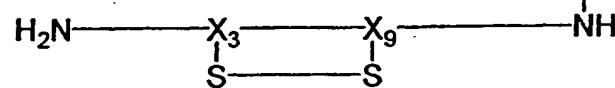
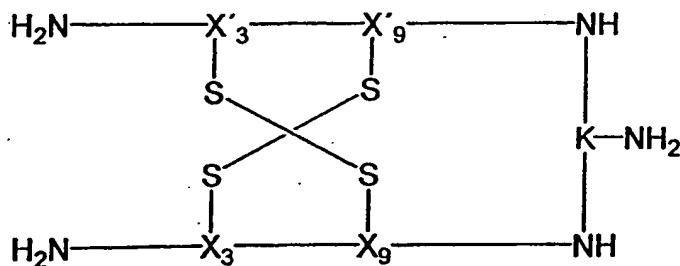
ISOMER 1ISOMER 2ISOMER 3

Figure 4

Ba/F3-huEPOR 06_10_98

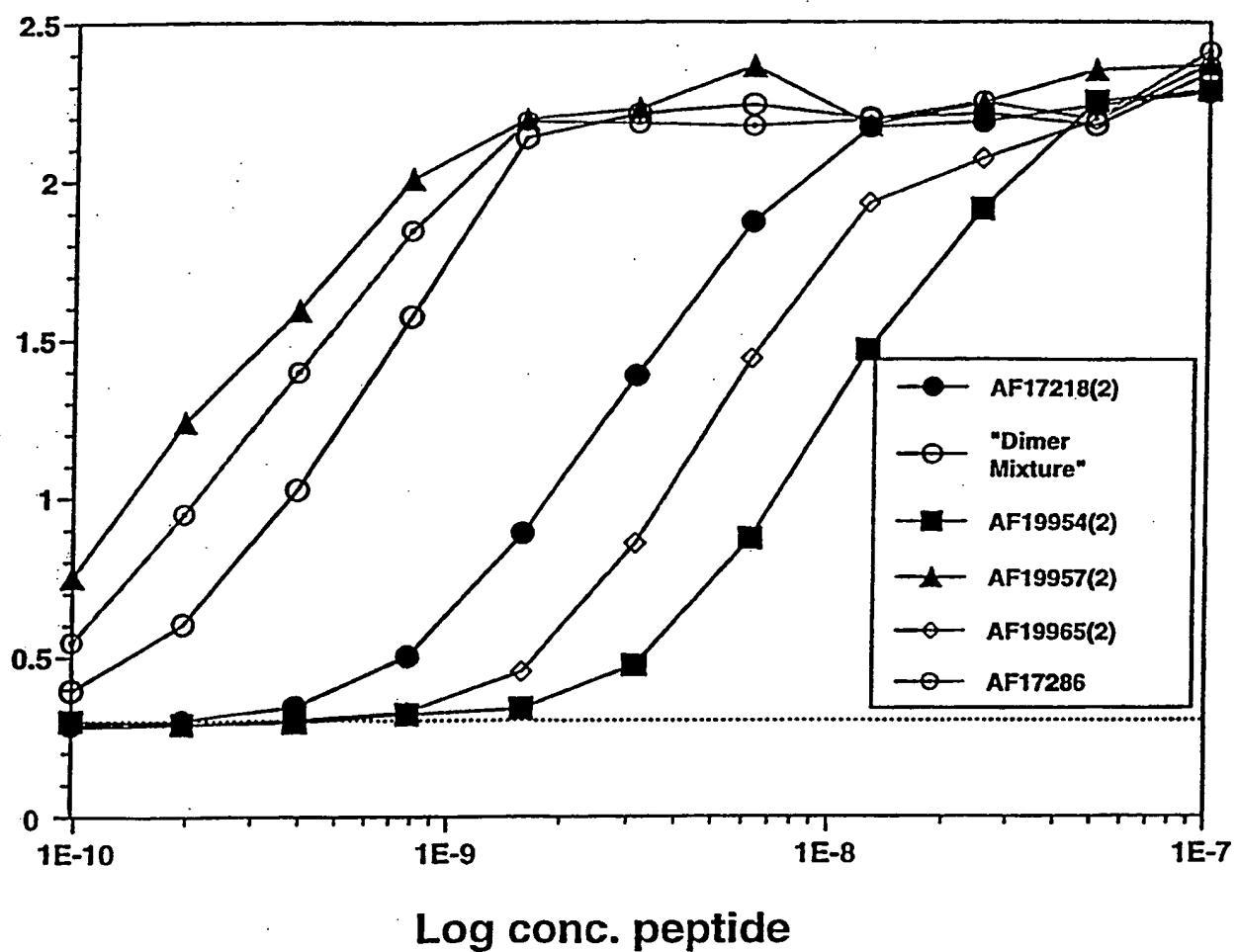


Figure 5